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TITLE: Role of Nkx3.1 Homeodomain Protein in Prostate Carcinogenesis and Differentiation

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Cancer of the prostate is the second most common cause of cancer morbidity and mortality in American males, currently accounting for ~30% of new cancer cases in this population. The chromosomal region most frequently involved in prostate cancer is located at 8p12-22 (3-5); indeed, a recent study showed that 63% of premalignant PIN foci have suffered chromosome deletions at this locus. One strong candidate for a gene involved in prostate cancer at this locus is the recently described Nkx3.1 gene, which encodes a homeodomain transcription factor. Studies of Nkx3.1-deficient mice have shown that the loss of a single allele is sufficient to promote hyperplasia in the prostate epithelium, indicating its potential role in prostate cancer initiation. To identify genes regulated by Nkx3.1, we have infected prostate carcinoma cells with adenovirus expressing Nkx3.1 and analyzed the changes in gene expression using microarray methodology. Immunohistochemistry using anti-Nkx3.1 antibodies was used to demonstrate that Nkx3.1 is expressed in luminal epithelial cells but not in basal, neuroendocrine or stromal cells. We examined Nkx3.1 expression in human prostate tumors and found that it was absent in the malignant cells in 60% of the tumor samples.

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Introduction. The central purpose of this project was to ascertain the role of the Nkx3.1 homeodomain transcription factor in prostate cancer through its control of prostate cell differentiation and/or proliferation processes. The Nkx3.1 gene is located on chromosome 8p21, a region likely to contain a prostate cancer tumor suppressor gene(s) as a majority of prostate carcinomas show loss of heterozygosity at this site. Nkx3.1 is a member of a class of homeodomain proteins that are crucial regulators of development and organogenesis. Nkx3.1 is expressed in the prostate anlage (or prostatic buds), and later, in prostate epithelial cells, suggesting that it may be an important factor in both initiating and maintaining prostate epithelial cell differentiation. The hypothesis is that Nkx3.1 is crucial for normal prostate cell development and differentiation. Therefore, loss of Nkx3.1 expression leads to a de-differentiation of prostate epithelial cells and subsequently to development of prostate carcinoma.

Body. The loss of Nkx3.1 is likely to be important in the initiation of prostate cancer based on the nature of the protein and the frequent loss of one allele in PIN lesions. Furthermore, recent evidence from our lab derived from studies of Nkx3.1-deficient mice suggests that loss of a single Nkx3.1 allele in adulthood is sufficient for the development of hyperplasia and dysplasia of the prostate epithelium. For this reason, it is important to identify genes that are regulated by the Nkx3.1 transcription factor in prostate epithelial cells. Towards this end (and to examine whether Nkx3.1 alters cell phenotype), we attempted to develop cell lines in which Nkx3.1 was overexpressed. We attempted these experiments in both fibroblasts, prostate cancer cell lines, and embryonic stem cells. We found that it was not possible to obtain stable cell lines that overexpress Nkx3.1, presumably because it inhibits cell proliferation. We have therefore implemented the GeneSwitch system, which allows the regulated expression (in response to mifepristone) of a particular gene product. This system has been problematic, but initial experiments with Nkx3.1 regulable expression in LnCAP cells indicate that these cells will not proliferate in the presence of high levels of Nkx3.1. This is consistent with the hypothesis that Nkx3.1 is important for differentiation.

Because the inability to develop Nkx3.1 overexpressing cell lines precluded there use in the identification of Nkx3.1-regulated target genes, we developed a novel system expressly for this purpose using a viral delivery system and microarray profiling. To help develop and test this strategy we used an adenoviral delivery system to overexpress the zinc finger transcription factor, Egr1, in the human prostate carcinoma cell line LAPC4, which retains many of the characteristics of normal prostate cells (PSA expression, androgen-dependent growth). The adenovirus expressing Egr1 construct was produced using the AdTrac system. The virus included an EGFP tag that allows coexpression of EGFP and subsequent visualization of postive infected LAPC4 cells. Using fluorescent microscopy for EGFP visualization, we have optimized the conditions of adenoviral delivery to show >95% positively infected LAPC4 cells. RNA was purified from cells at various times after infection, reverse transcribed and gene expression was determined by quantitative RT/PCR analysis using a TaqMan instrument. We found that a number of previously reported Egr1 target genes including IGF-II, TGF-β, and PDGF-A were up-regulated in the Egr1 infected cells, indicating that the procedure could be used to identify target genes.

To identify potential target genes that are regulated by Nkx3.1 in prostate carcinoma cells, we constructed an adenovirus expressing Nkx3.1. We used this virus (and a control

virus expressing only EGFP) to infect LAPC4 carcinoma cells. RNA was purified from these two sets of cells (24 hr after infection) and probes for Affymetrix Gene Chip analysis were synthesized by standard methods. These arrays (HuGeneFL chips (Affymetrix) contain probe sets for approximately 12,000 human genes. We analyzed the GeneChip result using Affymetrix software as well as the GeneSpring and Spotfire program suites, which contain programs useful for clustering gene expression data into self-organizing or hierarchical maps. In this manner, we have identified a number of differentially expressed genes between these two different sets of infected cells. Further analysis using quantitative RT/PCR analysis (TaqMan) revealed that their expression is indeed regulated by Nkx3.1. We also confirmed the Nkx3.1 regulation of these genes by analyzing prostates from Nkx3.1-deficient mice. Through these gain- and loss-of-function expression profiling experiments, we have found a number of very interesting genes that are regulated by Nkx3.1. These include growth-related proteins such as Chop-10 and B-myb, signaling proteins like RhoB, secreted molecules like angiogenin, PSA, mammoglobin B, fibromodulin, protein processing enzymes like Tom1 and Hsp68, and amino acid and ion transporters. Many of these proteins have been implicated in abnormal growth or differentiation in other systems, thus they may be involved in the abnormal growth control observed in prostate epithelial cells lacking Nkx3.1.

To examine Nkx3.1 expression in tissues, we produced anti-Nkx3.1 antibodies. Nkx3.1 protein was synthesized in bacteria using the pET system. This protein was used as an immunogen and injected into rabbits to generate anti-Nkx3.1 antisera. The anti-Nkx3.1 antibodies were purified by affinity chromatography using the bacterially synthesized Nkx3.1. These antibodies were tested for reactivity using protein blot analysis and immunohistochemistry using CV1 cells transiently transfected with an Nkx3.1 expression vector. After confirmation that the antibody was specifically detecting Nkx3.1 (both human and mouse), we used these antibodies to study expression of Nkx3.1 in the mouse prostate. We found that prostate expression of Nkx3.1was localized to the luminal epithelial cells and was not detected in basal, neuroendocrine or stromal cells. To examine the androgen-dependent expression of Nkx3.1 *in vivo,* we examined the expression of Nkx3.1 after castration. We found that Nkx3.1 expression was progressively lost in the prostate within 6 days following castration, consistent with its regulation by androgens. To examine whether testosterone administration would re-induce Nkx3.1 expression in the castrated prostate, we gave mice testosterone proprionate implants (and BrdU) for 3-4 days beginning 7 days after castration. We found that Nkx3.1 expression was restored within 4 days of testosterone replacement. We also examined the proliferative state of the Nkx3.1-positive cells using BrdU immunhistochemistry. Interestingly, we found that Nkx3.1 was not expressed in proliferating epithelial cells; instead, Nkx3.1 was only detected in non-proliferating prostate cells, consistent with our hypothesis that Nkx3.1 is important for prostate cell differentiation.

We also used the anti-Nkx3.1 antibodies to examine its expression in a series of human prostate tumors (n=20). We found that Nkx3.1 was expressed exclusively in the luminal cells of the prostate. Interestingly, Nkx3.1 expression was absent in the malignant cells in 60% of the tumor samples we analyzed. We hypothesized that loss of Nkx3.1 expression could be due to a mutation in the Nkx3.1 gene. To determine the state of the Nkx3.1 gene in prostate tumors, we isolated DNA from the malignant glands of 12

different prostate tumors and sequenced both exons of the Nkx3.1 gene. We did not identify any mutations in the Nkx3.1 gene in the 12 samples we examined, a finding that has been independently confirmed by others. Thus, the absence of Nkx3.1 expression in prostate tumor cells is likely the result of altered expression, either due to mutation or hypermethylation of the promoter region.

We also hoped to produce prostate stem cell lines using the Nkx3.1 locus to drive an oncogene. Unfortunately, this portion of the project was not completed. We cloned the Nkx3.1 locus in a BAC clone and sequenced a large portion of the locus including several thousand base pairs of the promoter region. We attempted to identify a region of the Nkx3.1 promoter that would drive expression of transgenes in a pattern that recapitulates expression of the endogenous Nkx3.1 gene. For this purpose we cloned an EGFP reporter downstream of a promoter fragment and transfected this construct into prostate carcinoma cells lines, but no expression was observed. To pursue this project further will require much larger fragments of the Nkx3.1 locus in order to achieve faithful expression. If a useful fragment could be identified using cell lines, then it could be used to construct transgenic mice. If faithful expression of the EGFP reporter was obtained in vivo, then this fragment could be used to drive SV40 Tantigen and prostate stem cells could potentially be derived. However, with the exciting advances in identifying stem cells from a variety of human and mouse tissues, including brain, liver, muscle, etc, it is likely prostate stem cells will soon be isolated using similar methods without the necessity for transgenic technology.

Key Research Accomplishments.

- We expressed Nkx3.1 in a number of cell types and found that it appears to inhibit cell proliferation.
- We developed a novel system for identifying target genes of any transcription factor using an adenoviral delivery system coupled with microarray gene expression profiling technology. This allowed us to identify a number of genes that are regulated by Nkx3.1 in prostate epithelial cells.
- We expressed Nkx3.1 in bacteria and used this protein to produce antisera in rabbits that was demonstrated to be specific for Nkx3.1. This antisera was used to show that Nkx3.1 expression is lost in approximately 60% of human prostate tumors.
- Using laser-capture microdissection, we isolated DNA from human prostate specimens. Both exons of the Nkx3.1 gene were sequenced in these specimens but no mutations were identified, thus mutation of the Nkx3.1 gene is not a common event in prostate cancer.
- Using immunohistochemistry we determined that Nkx3.1 is expressed in the non-proliferating luminal epithelial cells of the prostate and not in surrounding basal, neuroendocrine or stromal cells. After castration, Nkx3.1 expression is lost. Upon administration of testosterone, Nkx3.1 expression returns but it is only expressed in cells that have ceased proliferating.

Reportable outcomes. LnCAP cells expressing regulable Nkx3.1; Adenovirus expressing Nkx3.1; Production of anti-Nkx3.1 antibodies that recognize human and

mouse Nkx3.1; Identification of Nkx3.1 target genes; John Svaren, Ph.D. obtained an Assistant Professorship at the University of Wisconsin

Conclusions. Based on evidence gathered here, it appears that loss of Nkx3.1 is important for initiation of prostate cancer, presumably through its role as a tumor suppressor. The finding that Nkx3.1 expression is lost in a majority of prostate tumors supports this idea. The fact that no mutations in Nkx3.1 were found in these tumors, suggests that its expression is curtailed by another mechanism, perhaps through hypermethylation of the promoter. The identification of genes regulated by Nkx3.1 via gene expression profiling provides a useful tool for pursuing the mechanism by which Nkx3.1-deficiency results in abnormal prostate epithelia cell growth. Our observations on Nkx3.1 expression in mice confirm the idea that Nkx3.1 is important in maintaining the differentiated state of prostate epithelial cells, a finding consistent with our finding that Nkx3.1 overexpression inhibits cell proliferation.

References. None

Appendices: None

Final Reports - Personnel receiving pay from the research effort:

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